REMARKS

In the amendments above, Claims 1-7 have been cancelled and new Claims 8-19 have been added, to more particularly point out and distinctly claim the Applicants' invention. Support for new Claims 8 and 14 may be found on page 6, lines 16-20; page 11, lines 3-11; and lines 27-34 of the specification. Support for new Claims 9 and 15 may be found on page 6, lines 3-5, and page 11, lines 12-14 of the specification. Support for new Claims 10 and 16 may be found on page 6, lines 3-5, and page 11, lines 15-17, of the specification. Support for new Claims 11 and 17 may be found on page 6, lines 3-5, and page 11, lines 18-20, of the specification. Support for new Claims 12 and 18 may be found on page 6, lines 3-5, and page 11, lines 21-23, of the specification. Support for new Claims 13 and 19 may be found on page 6, lines 3-5, and page 11, lines 24-26, of the specification.

35 U.S.C. §101 Rejections

Claims 1-7 have been rejected under 35 U.S.C. § 101. The Examiner maintains that the claimed recitation to use, without setting forth any steps involved in the process results in an improper definition of a process, i.e., results in a claim which is not a proper process claim under 35 U.S.C. § 101.

Applicants have cancelled Claims 1-7 directed to the use of the compound of the invention and have added new Claims 8-19, compliant with 35 U.S.C. § 101, directed to methods of treatment and methods of prevention. Applicants respectfully request the Examiner reconsider and withdraw the rejection under 35 U.S.C. § 101.

35 U.S.C. §112, Second Paragraph Rejections

Claims 1-7 have been rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. The Examiner maintains that Claims 1-7 provide for the use of a composition including myo-inositol hexaphosphate in a form

adapted to topical administration for the manufacture of a formulation for the prevention and/or treatment of a disease associated with the development of heterogenous nucleants which induce the development of pathological calcification in a soft tissue. The Examiner states that the term "and/or" in Claim 1 is indefinite and could reasonably be construed to have two mutually exclusive different meanings.

Applicants have cancelled Claims 1-7 and added new Claims 8-19 above, which claims do not recite the term "and/or." Applicants respectfully request the Examiner reconsider and withdraw the rejections under 35 U.S.C. § 112, second paragraph.

35 U.S.C. § 102(b) Rejection

Claims 1-7 have been rejected under 35 U.S.C.§102 (b) as being anticipated by Znaiden et al., U.S. Patent Application 5,268,176 ("Znaiden"). The Examiner maintains that Znaiden teaches topical compositions containing inositol hexaphosphate (phytic acid or myo-inositol) for use in the treatment of telangiectasia characterized by visual dilation of one or more superficial skin arterioles in the human body. The Examiner states that Claim 1 recites a composition including the identical active compound as taught by Znaiden, i.e., myoinositol, for use in the prevention and/or treatment of a disease associated with the development of heterogenous nucleants which induce the development of pathological calcification in a soft tissue. The Examiner maintains that to the extent that Claim 1 recites the identical active compound for topical administration to a soft tissue, the contemplated treatment effect of the instant invention is deemed to be an inherent characteristic of topically administering the identical composition. The Examiner further maintains that the limitations found in Claim 2-7 are also considered to be inherent features.

Applicants initially note that anticipation requires that each and every element of the claims be disclosed, either expressly or inherently, in a single prior art reference or embodied in a single prior art device or practice. *See* In re Paulsen, 30 F.3d 1475, 1478 (Fed. Cir. 1994); Minnesota Min. & Mfg. Co. v. Johnson & Johnson Orthopaedics, Inc.,

976 F.2d 1559, 1565 (Fed. Cir. 1992). There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of invention. *See* Scripps Clinic & Res. Found. v. Genentech, Inc., 927 F.2d 1565, 1576 (Fed. Cir. 1991). A finding of anticipation "is not supportable if it is necessary to prove facts beyond those disclosed in the reference in order to meet the claim limitations." *Id.*

Znaiden only mentions the use of phytate on the skin. The phytate as taught by Znaiden is used specifically over spider veins and does not mention or suggest the capacity of phytate for being absorbed through the skin, passing into the bloodstream, and acting on pathological calcification. For example, on Column 3, lines 7-11, of Znaiden, it is stated that "[t]he hydroxyl groups may allow the molecule to readily penetrate the most superficial layers of skin which are slightly polar because they lack a significant phospholipid concentration." Column 3, lines 18-24, recites:

> [o]nce a highly polar molecule reaches viable tissue, it will be repulsed by cell membrane phospholipids and remain in the intracellular space. Instead of being lost through dissipitation, the molecules remain sequestered and form a stable depot, which creates a high osmotic gradient necessary for the collapse of an offending vessel.

The myo-inositol of the present invention is not limited to an active location on the surface of the skin. Instead, the myo-inositol is absorbed by the skin and enters the patient's bloodstream. On page 5, lines 15-20, of the specification, it is stated that "[s]urprisingly, the inventors of this invention have found that phytate, with a high negative charge, can be absorbed by the skin when it is administered topically, passing through into the bloodstream and acting on the damaged zone (in which a heterogeneous nucleant would have been generated)."

As Znaiden does not teach a method of treatment or prevention wherein the phytate is absorbed through the skin into the bloodstream, as disclosed in new Claims 8-19, and does not disclose every element of the claims, Applicants respectfully request the Examiner reconsider and withdraw the rejection under 35 U.S.C. §102(b).

35 U.S.C. § 112, First Paragraph

Claims 1-7 have been rejected under 35 U.S.C. § 112, first paragraph, because the specification, while enabling for methods of preparing and methods of use of myo-inositol compositions for treating certain diseases associated with the development of heterogenous nucleants which induce the development of pathological calcification in a soft tissue, does not reasonably provide enablement for preventing said diseases and/or treating any and all diseases associated with the development of heterogenous nucleants which induce the development of pathological calcification in a soft tissue. The Examiner stated that to be enabling, the specification of the patent application must teach those skilled in the art how to make and use the full scope of the claimed invention without undue experimentation.

The Examiner maintains that the invention in general relates to a use of myo-inositol compositions for topical administration for treating diseases associated with the development of heterogeneous nucleants which induce the development of pathological calcification in a soft tissue. The Examiner maintains that Grases et al. (Grases et al., "Effect of Crystallization Inhibitors on Vascular Calcifications induced by Vitamin D: A Pilot study in Sprague-Dawley rats. Cir. J. 2007:71:11-52-1156) ("Grases") teach that pathological calcification in soft tissues (i.e., ectopic calcification) can have severe consequences when it occurs in vital organs such as the vascular or renal systems. The Examiner further maintains that Grases teaches, generally, that the development of tissue calcification requires a pre-existing injury as an inducer, whereas further progression requires the presence of other promoter factors (such as hypercalcemia and/or hyperphosphatemia) and/or a deficiency in calcification repressor factors. The Examiner maintains that Grases teaches that pyrophosphate, biphosphonates and phytate have been shown to inhibit crystallization in the form of vascular calcification and that Grases also teaches that based on the fact that phytates were found to act as vascular calcification inhibitors, the action of polyphosphates could be important in protecting against vascular calcification.

The Examiner further maintains that the claims are broad in scope. The Examiner stated that the disclosure does not provide any definition of the term "heterogenous nucleants," or does not disclose the connection between the administration of myo-inositol hexaphosphate and its effect on heterogenous nucleants, or how the effect on heterogenous nucleants relates to the contemplated effects to be achieved in practicing the instant invention. The Examiner further noted that Claim 1 recites the term "pathological calcification in a soft tissue," which encompasses pathological calcification in soft tissues of any and all mammalian species. The Examiner maintains that because the therapeutic response to be achieved would reasonably vary depending upon the specific mammalian species, targeted soft tissue, location of the soft tissue, and the pharmacodynamic/pharmacokinetic profile of myoinositol hexaphosphate, the level of predictability in practicing the claimed invention would be greatly diminished.

The Examiner further notes that the specification discloses a study involving the topical administration of phytate to rats. The Examiner maintains that based on the instant disclosure, Applicants have provided specific direction or guidance only for a general method of using a myo-inositol composition. The Examiner stated that extrapolation of the exemplified rat data disclosed by Applicants to any and all mammalian species would reasonably require extensive experimentation in order to achieve the contemplated treatment effects in practicing the instant claimed invention commensurate with the claims.

The Examiner further maintains that in view of the uncertainty and unpredictability of the art as evidenced by the discussion of the prior art, it is reasonable to surmise that this level of uncertainty in the art would require one skilled in the art to conduct more than routine experimentation in order to practice the claimed invention commensurate with the scope of the claims.

Applicants initially note that Claims 1-7 have been cancelled and replaced with new Claims 8-19. In the new claims, "heterogeneous nucleant" has been replaced by

"calcification" based on page 6, lines 9-15, which states "[t]hese analysis models therefore indicate that a composition including phytate in a form adapted to topical administration can be used for the manufacture of a medicament for this treatment of a disease associated with the formation of heterogeneous nucleants, preferably of a disease associated with the formation of calcifications, in a soft tissue."

Applicants further note, with regard to the term, "calcification in soft tissue," the Examiner was doubtful about which soft tissues and species can be included under the present treatment since there are a large variability of parameters. Applicants herewith attach six references, published after the priority date of the present application, showing the possibility of using topically administered phytate in the treatment of calcification in several types of tissues. The attached references prove that a therapeutic response occurs at each of the claimed soft tissue locations noted in the application and further that extensive experimentation was not necessary to achieve the claimed treatment effects in mammals other than rats. Grases et al., "Study of a myo-inositol hexaphosphate-based cream to prevent dystrophic calcinosis cutis," *British Journal of Dermatology* 2005:152, pp 1022-1025, ("**Document 1**") establishes the effects of topically administered myo-inositol hexaphosphate on subepithelial cells.

Grases et al., "Phytate acts as an inhibitor in formation of renal calculi," *Frontiers in Bioscience* 12, 2580-2587, January 1, 2007, ("**Document 2**") describes the use of a topically administered phytate with on renal calcifications. Topically administered phytates may also be used for treatment of calcification of cardiovascular tissue. For example, Grases et al., "Phytate (Myo-inositol hexakisphosphate) inhibits cardiovascular calcification in rats," *Frontiers in Bioscience* 11, 136-142, January 1, 2006, ("**Document 3**") describes a topically administered phytate which is effective to decrease mineral deposits in hearts and arteries.

Applicants further submit Grases et al., "Study of the Absorption of Myo-Inositol Hexakisphosphate ($InsP_6$) through the Skin," *Biol. Pharm. Bull.* 28(4):764-767, 2005,

("**Document 4**") and Grases et al., "Absorption of myo-inositol hexakisphosphate ($InsP_6$) through the skin in humans," *Pharmazie* 61(7): 652, 2006, ("**Document 5**"). Documents 4 and 5 demonstrate that phytate can be topically absorbed in rats and humans.

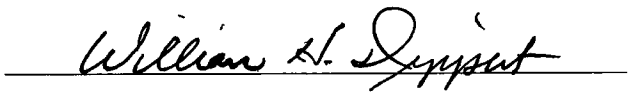
The Examiner further stated that extension of the present application to any and all mammalian subjects, including humans, would require extensive experimentation. Applicants note that both Document 5 and Grau et al., "Relationship Between Phytate and Valvular Heart Calcifications. Field Study To Design a Clinical Assay About Aortic Stenosis" ("**Document 6**") disclose the topical use of a phytate on humans. Documents 5 and 6 show that the primary results obtained with rats can be extrapolated to other species without the need for extensive experimentation.

In view of the comments above and the amendments to the claims, Applicants respectfully request the Examiner withdraw the rejections of the Claims under § 112, first paragraph.

Reconsideration and allowance of all the claims herein are respectfully requested.

Respectfully submitted,

February 5, 2008



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Study of a *myo*-inositol hexaphosphate-based cream to prevent dystrophic calcinosis cutis

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Conflicts of interest:
None

Summary

Background Calcinosis cutis is a disorder caused by abnormal deposits of calcium phosphate in the skin and is observed in diverse disorders. *Myo*-inositol hexaphosphate ($InsP_6$) is a diet-dependent molecule found in all mammalian fluids and tissues, which exhibits an extraordinary capacity as a crystallization inhibitor of calcium salts.

Objectives To establish the effects of topically administered $InsP_6$ cream on artificially provoked dystrophic calcifications in soft tissues.

Methods Fourteen male Wistar rats were randomly assigned into two groups: control and treated groups. Rats were fed with an $InsP_6$-free or phytate diet. Plaque formation was induced by subcutaneous injection of 0·1% $KMnO_4$ solution. From 4 days before plaque induction to the end of the experiment, control rats were treated topically with a standard cream, whereas treated rats were treated with the same cream with 2% $InsP_6$ or phytate (as sodium salt). Calcification of plaques was allowed to proceed for 10 days. $InsP_6$ in urine was determined. The plaques were excised and weighed.

Results It was found that when $InsP_6$ was administered topically through a moisturizing cream (2% $InsP_6$-rich), the plaque size and weight were notably and significantly reduced compared with the control group (1·6 ± 1·1 mg $InsP_6$-treated, 26·7 ± 3·0 mg control). The $InsP_6$ urinary levels for animals treated with the $InsP_6$-enriched cream were considerably and significantly higher than those found in animals treated topically with the cream without $InsP_6$ (16·96 ± 4·32 mg L^{-1} $InsP_6$-treated, 0·06 ± 0·03 mg L^{-1} control).

Conclusions This demonstrates the important capacity of $InsP_6$ as a crystallization inhibitor and also demonstrates that it is possible to propose topical use as a new $InsP_6$ administration route.

Calcinosis cutis is a disorder caused by an abnormal deposit of calcium phosphate in the skin and is observed in a number of disorders;[1–4] a significant incidence of cutaneous calcifications has been identified in dermatomyositis[5,6] and systemic sclerosis.[7,8] On occasion, this disorder may have an iatrogenic origin which can be caused by subcutaneous and/or intramuscular injections.[9–11] It is now known that several factors contribute to the formation of calcium deposition in soft tissues and among them lack of the so-called crystallization inhibitors must be considered. These are substances that due to their chemical structure interact with the nucleus or crystal faces provoking important disturbances in their formation and/or development, consequently preventing crystallization processes. Despite the well-known fact of the existence of crystallization inhibitors and the importance of their benefits, this is still a scarcely studied phenomenon in biological processes and, in fact, relatively few new papers appear each year. At present few inhibitor molecules are of clinical use. The first biological crystallization inhibitor to be recognized and studied was pyrophosphate, around the 1960s.

Myo-inositol hexaphosphate ($InsP_6$, phytate) is a molecule found in abundance in plant seeds (~ 1·5–6·4%). In most seed types the $InsP_6$ is associated to calcium and magnesium ions, forming a salt named phytin; it is not distributed equally among the seed parts. For example, the endosperm of wheat and rice kernels is almost devoid of phytate as it is concentrated in the germ and aleuronic layers of cells of the kernel and in the bran or hull. Corn differs from most cereals as almost 90% of phytate is concentrated in the germ portion of the kernel as in the carob germ.

$InsP_6$ is also found in all mammalian organs, tissues and fluids, but at significantly low concentrations.[12,13] The levels found in blood and mammalian tissues clearly depend on dietary intake.[12–14] Moreover, it has been demonstrated that this molecule exhibits extraordinary capacity as a crystallization inhibitor of calcium salts in urine and soft tissues.[15,16] The $InsP_6$ present in urine exhibits an important role on preventing renal calculi.[14,16,17] Other important physiological functions of $InsP_6$ have been suggested, such as its antioxidant properties[18,19] and its role in colon cancer prevention.[20,21] Also, recent studies have demonstrated the seeming capacity of $InsP_6$ to prevent skin cancer.[22,23]

The aim of this paper is to study the effects of topically administered $InsP_6$ cream on artificially provoked dystrophic calcifications in soft tissues. The $InsP_6$ sodium salt was chosen over the $InsP_6$ calcium–magnesium salt due to the higher water solubility of the sodium salt. This study will also allow the evaluation of the capacity of $InsP_6$ penetration into the organism through the skin.

Materials and methods

Animals, diets and experimental design

Fourteen male Wistar rats weighing 275–300 g from Harlan Iberica s.l. (Barcelona, Spain) were acclimated over the course of 7 days to our animal house. Animals were kept in Plexiglas™ cages (two animals per cage) at a temperature of 21 ± 1 °C and relative humidity of 60 ± 5% with a 12-h on–off light cycle. After this period, animals were randomly assigned into two groups of eight (control group) and six (treated group) rats. Rats were fed with the 4068.02 Reference Diet (HopeFarms BV, Woerden, the Netherlands), a synthetic purified diet (Table 1) in which $InsP_6$ is undetectable.

Table 1 Composition of 4068·02 Reference Diet, a synthetic purified diet in which $InsP_6$ is undetectable

Ingredient	% in diet
Acid casein	20
Corn starch	10
Cellulose	15
Soybean oil	5
Glucose	52·8
$CaPO_4 \cdot 2H_2O$	1·3
$CaCO_3$	1·7
KH_2PO_4	0·40
KCl	0·70
NaCl	0·30
$MgSO_4 \cdot 7H_2O$	0·40
MgO	0·20
DL-methionine	0·20
Choline chloride	0·40
Standard vitamin mix	1
Standard mineral mix	1

Each experimental group was fed this diet for 31 days and animals ceased to consume the diet just before killing. After a period of 21 days, plaque formation was induced by subcutaneous injection of a 0·1% $KMnO_4$ solution (200-μL dose) in two positions on either side of the interscapular region. From 4 days before plaque induction to the end of the experiment, control rats were treated topically with 1 g of a standard cream twice daily, whereas the treated group was treated topically twice daily with 1 g of the same standard cream supplemented with 2% $InsP_6$ as sodium salt. The treatment surface was about 50 cm^2 and was shaved before cream application. The pH of both creams was adjusted to 4–4·5 (see Table 2). Calcification of plaques was allowed to proceed for 10 days; after this period, the animals were anaesthetized with pentobarbital (50 mg kg^{-1}, intraperitoneally); then, the plaques were excised and weighed. Urine samples (24-h) were also collected the day before killing. It was established previously that there is a direct relationship between plaque weight and precipitation of hydroxiapatite.[24,25] The procedures used in this experiment were carried out according to Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes and official permission was obtained from the ethics committee of the University of Balearic Islands.

Cream preparation

The moisturizing $InsP_6$ cream (O/W) was prepared using two different phases, oil and water (see Table 2). The oil phase (O) and water phase (W) were each preheated to

Table 2 Composition of both moisturizing creams used

Component	Composition (%)	
	$InsP_6$-enriched cream	Standard cream
Oil phase (O)		
Almond oil	4	4
Glyceril stearate	4	4
Cetearyl alcohol	4	4
Isopropil miristate	3·8	3·8
Vitamin E	2·5	2·5
Lactic acid	1·6	1·6
Stearic acid	1	1
Propyl paraben	0·1	0·1
Controx VP	0·03	0·03
Water phase (W)		
Water	70	72·9
Glycerine	4·87	4·87
Sodium $InsP_6$*	2·9	0
Laurylate 5·90	0·3	0·3
Imidazolidinil urea	0·3	0·3
Essence	0·3	0·3
Methyl paraben	0·2	0·2
Triethanolamine	0·1	0·1

*2·9% of sodium $InsP_6$ corresponds to 2·0% of $InsP_6$.

65 ± 5 °C and were then mixed to obtain the O/W emulsion. For this purpose the W phase was slowly added to the O phase, with intensive stirring and homogenizing throughout the process. The emulsion was then stirred intensively for 10 min at 65 ± 5 °C and finally cooled at room temperature, maintaining the intensive stirring for the duration.

$InsP_6$ determination

The determination of $InsP_6$ levels in urine samples was performed using an analytical methodology based on determining total phosphorus by inductively coupled plasma atomic emission spectrometry.[26] This method allows a measurement of total $InsP_6$ with a detection limit of 60 μg L^{-1}.

Statistics

Values given in the tables and figures are expressed as mean (SE). Student's t-test was used to assess differences of means. Conventional Windows software was used for statistical computations. A value of $P < 0.05$ was considered to assess statistical significance.

Results

The results obtained about the hydroxiapatite plaques from the control and treated groups are shown in Figures 1 and 2. As can be observed, when $InsP_6$ was topically administered through the cream, the plaque size and weight were notably and significantly reduced. Thus, for animals treated topically with the $InsP_6$-enriched cream (with 2% $InsP_6$) the mean plaque weight was 1·6 ± 1·1 mg, whereas for animals treated topically with the same cream without $InsP_6$, the mean plaque weight was 26·7 ± 3·0 mg.

Table 3 shows the $InsP_6$ levels found in the urine of the treated and control groups. As can be observed, for animals treated with the $InsP_6$-enriched cream, $InsP_6$ levels were considerably and significantly higher than those found in animals treated topically with the cream without $InsP_6$.

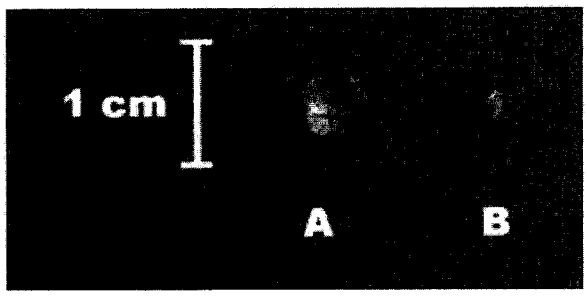

Fig 1. Representative image of hydroxiapatite plaques from the two groups. (A) Control group: animals treated with 1 g of a standard cream twice daily. (B) Treated group: animals treated with 1 g of a standard cream with a 2% of $InsP_6$ as sodium salt, twice daily.

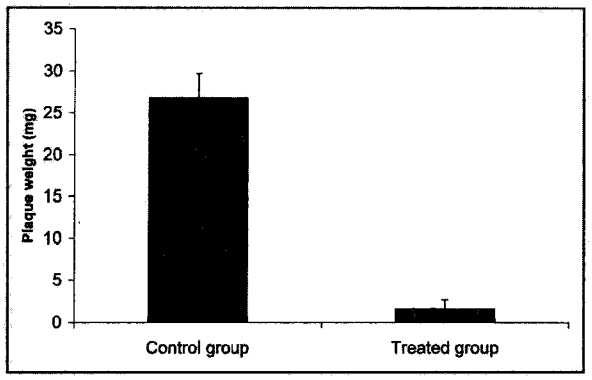

Fig 2. Chemically induced calcified plaques. Data are expressed as mean of dry plaque weight (mg) for each group. Control group ($n = 8$): animals treated with 1 g of a standard cream twice daily. Treated group ($n = 6$): animals treated with 1 g of a standard cream with a 2% of $InsP_6$ as sodium salt, twice daily. Error bars represent SE (*$P < 0.001$).

Table 3 Concentration of $InsP_6$ in urine.

Group	n	$[InsP_6]_{urine}$ (mg L^{-1})
Control	8	0·06 (0·03)
Treated	6	1896 (492)*

The results are expressed as mean (SE) of concentration (mg L^{-1}). Control group: animals treated with 1 g of a standard cream twice daily. Treated group: animals treated with 1 g of a standard cream with 2% $InsP_6$ as sodium salt, twice daily. *$P < 0.001$.

Discussion

All extracellular mammalian fluids are supersaturated with regard to calcium phosphate (hydroxiapatite). Consequently, they are metastable in relation to the formation of this solid. Nevertheless, these crystals do not spontaneously appear. Physiologically, crystallization takes place only in controlled situations such as in bone or tooth formation. However, uncontrolled pathological crystallizations are also frequent. In fact, crystallization does not indiscriminately take place in all biological fluids because it depends not only on thermodynamic factors (supersaturation) but also on kinetic factors. Thus, biological calcifications depend mainly on three factors: the supersaturation (thermodynamic factor), the presence of heterogeneous nucleants (crystallization promoters, kinetic factor) and/or crystallization inhibitors (kinetic factor). The presence of injured tissue supplies heterogeneous nucleants that act as substrates for the initial crystal formation.[27] The action of crystallization inhibitors can prevent or reduce the crystal formation.

The results of this paper demonstrate, on the one hand, that $InsP_6$ was absorbed through the skin, crossing the dermis and epidermis, entering the bloodstream and increasing the urinary excretion. In this aspect, it is interesting to observe how the urinary levels attained were higher than those observed in

rats consuming an $InsP_6$-rich standard diet.[12,13] It must be considered that tissue and fluid levels derived from dietary $InsP_6$ are limited by its rate of absorption from the gastrointestinal tract. There appears to be an optimum ingested amount, above which no further absorption occurs;[28] this amount was found to be 20·9 mg kg^{-1} day^{-1} for animals.[29] Extrapolating this to a human subject of 70-kg body weight, the minimum intake to obtain maximum absorption was calculated to be 1463 mg and was independent of the type of $InsP_6$ salt consumed.[28] The normal urinary level found in rats consuming an $InsP_6$-rich standard diet was ~3·02 ± 0·09 mg L^{-1}.[16] On the other hand, the crystallization inhibitory capacity of $InsP_6$ against hydroxiapatite deposit development was clearly manifested. Thus, due to the $InsP_6$-deficient diet consumed, the control group developed considerable calcified plaques (Fig. 1). In fact, the group treated topically with $InsP_6$ was fed with the same $InsP_6$-deficient diet but, due to the absorption of this molecule through the skin, as demonstrated by the increased $InsP_6$ excretion, the size of the calcified plaques dramatically decreased. These results demonstrate the important capacity of $InsP_6$ as a crystallization inhibitor of pathological calcifications, with a clear potential to avoid cutaneous calcifications associated with diverse skin disorders. Finally, as a consequence of these results, it is possible to introduce the topical use as a new route of administration of $InsP_6$.

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Phytate acts as an inhibitor in formation of renal calculi

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1. ABSTRACT

The aim of this study was to assess the inhibitory action of phytate in formation of renal calculi. Hypertension (induced by nicotine) combined with hypercalcemia (induced by D vitamin) was used to induce calcification in renal tissue in male Wistar rats that were fed a purified phytate free diet. Phytate non-treated rats developed significant calcium deposits in kidneys and papillae, as well as in kidney tubules and vessels, whereas calcium deposits were absent in control and phytate treated rats. Fragments of hydroxyapatite (HAP) calculi exhibited the capacity to induce the growth of calcium salts on their surfaces. Presence of 1.5 mg/L of phytate in the synthetic urine inhibited the formation of calcium oxalate monohydrate on HAP renal calculi in normocalciuric conditions. The findings show that the action of phytate as a crystallization inhibitor takes place both in the intrapapillary tissue and urine.

2. INTRODUCTION

Pathological calcification is an undesirable process that frequently occurs in soft tissues. Development of calcification involves complex physicochemical and molecular biological events. Injury acts as an inducer of calcification (hydroxyapatite, HAP) but continuation of this process depends on presence of other promoter conditions (hypercalcemia, hyperphosphatemia) and/or the deficit of inhibitory factors (altered cellular calcification modulators, deficit of crystallization inhibitors).

Papillary renal calculi are small size uroliths, mainly composed of calcium oxalate monohydrate (COM). They exhibit a typical morphology consisting of a concave face (zone of union with the papillary tissue) and an opposite smooth convex face. According to recent studies, around 13% of all renal calculi are of renal papillary type (1). A COM papillary stone can only develop from a nidus

comprised of several crystals and/or organic matter that attach to the kidney papilla. This nidus can remain on site, in close contact with urine, and with an altered epithelium that resists nidus permanence. Urine remains supersaturated with calcium oxalate (2,3). HAP has been identified as the major component of the nidus (core) in 39.2% of papillary calculi (1). In the 1930's, Randall described a pre-calculus lesion in the renal papilla and proposed that a subepithelial calcification of renal papilla becomes the nidus of COM papillary calculi, as a consequence of the disruption of the papillary epithelial layer by the HAP plaque (4-10). Recently, it was found that in patients susceptible to the development of calcium renal calculi, plaque is initiated in thin-loop basement membranes, whereas in patients who have undergone bypass surgery, these plaques are formed in intratubular HAP crystals in renal collecting ducts (7-10).

Myo-inositol hexaphosphate ($InsP_6$, phytate) is abundant in plant seeds (~ 1.5-6.4%) and is present in all mammalian tissues and fluids, at a significantly low concentration (11,12). The levels found in blood and mammalian tissues are dependent on the amount in the dietary intake (11-13). Phytate inhibits crystallization of calcium salts in urine and soft tissues, and prevents the formation of renal calculi (13-16), acts as an antioxidant (17,18) and protects against development of colon cancer (19,20). The aim of this paper was to study the factors implied in formation of intrapapillary calcifications, to evaluate the capacity of HAP calcifications to promote crystallization of calcium salts in urine and to assess the inhibitory action caused by phytate in both cases.

3. MATERIAL AND METHODS

3.1.*In vivo* studies

3.1.1. Animals, diets and treatments

Eighteen male Wistar rats of approximately 250 g from Harlan Iberica S.L. (Barcelona, Spain) were acclimated in the course of 7 days. Animals were kept in Plexiglas cages (three animals per cage) at a temperature of 21 +/- 1°C and relative humidity of 60 +/- 5% with a 12-h on-off light cycle. After this period, animals were randomly assigned into three groups of six rats respectively: control group (subjected to placebo calcinosis induction), phytate non-treated group (subjected to calcinosis induction) and phytate treated group (subjected to calcinosis induction and pre-treated with a phytate containing cream). No control treated group with the moisturizing cream without phytate was included because it was previously found that such moisturizing cream without phytate had no effects on preventing dystrophic calcifications in rats (21). All rats were fed AIN 76-A diet (Ssniff Especialdiatën GmbH, Soest, Germany), a purified diet in which phytate is undetectable. The procedures used in this experiment were carried out according to the Directive 86/609/EEC for protection of animals and after obtaining permission from the Bioethical Committee of the University.

3.1.2. Phytate containing cream pre-treatment

After a period of 16 days on a AIN 76-A diet, urinary phytate became undetectable. Then, phytate treated group received once a day for the duration of the experiment (20 days), topical skin application of 4 g of a cream containing 2.0% potassium salt of phytate (22). The application skin surface (~50 cm^2) on the back was shaved every four days.

3.1.3. Cream preparation

The moisturizing phytate cream (O/W) was prepared using two different phases, oil and water phases according to Table 1. The oil phase (O) and water phase (W) were previously heated until 65 +/- 5 °C and then were mixed to obtain the O/W emulsion. For this purpose the W phase was slowly added to the O phase, with intensive stirring and homogenizing during all the process. Then the obtained system was intensively stirred for 10 min. at 65 +/- 5 °C and finally cooled at room temperature maintaining the intensive stirring during all time.

3.1.4. Induction of calcinosis, monitorization and sample acquisition

After a period of 16 days of receiving cream, phytate non-treated group and phytate treated group were subjected to calcinosis induction. Another control group was subjected to placebo calcinosis induction. Calcifications were induced according to P. Kieffer *et al.* (23) by intramuscular injection of 300.000 IU/kg of vitamin D_3 (supplied by Fort Dodge Veterinaria, S.A., Girona, Spain) and 25 mg/kg oral administration of (-)-nicotine hydrogen tartrate salt (5 g/L solution, Sigma Aldrich, Steinheim, Germany). Nicotine administration was repeated ten hours later. Control group received intramuscular injection of NaCl (0.15 M) and distilled water orally. Animals were monitored every 12 hours. After 60 hours of calcinosis induction, all rats of the phytate non-treated group died, and the rest of rats were sacrificed, and kidneys were removed.

3.1.5. Histological analysis

Tissues were fixed in 10% buffered formalin, embedded in paraffin and examined after hematoxylin and eosin staining. The extent of calcium deposits was scored semiquantitatively as absent, low, moderate or high.

3.1.6. Quantitation of phytate

Levels of phytate were quantified in the 24 hr urine samples collected on the day before calcinosis was induced by inductively coupled plasma atomic emission spectrometry (ICP-AES, Perkin-Elmer, USA.) following total phosphorus determination, which has a detection limit of 60 µg/L (24). To do this, 5.0 ml of urine (acidified with HCl 1:1 until pH = 3-4) was transferred to a column containing 0.2 g of anion exchange resin (AG 1-X8, Bio-Rad Laboratories, U.S.A.). The first eluate was discarded, the column was washed first with 50 ml of HCl 50 mM. The second eluate was discarded. Finally, the column was washed with 3.0 ml of HNO_3 2 M. The ICP-AES conditions were set as follows: outer argon flow 15 L/min, auxiliar argon flow 1 L/min, inner argon flow 1 L/min, nebulizer uptake rate 1 mL/min and wavelenght 213.618 nm. The determination of phytate was then carried out through direct phosphorus analysis of the last eluate by ICP-AES using a calibration curve.

Table 1. Composition of the moisturizing cream used

Component	Percentage composition
Oil phase (O)	
• Almond oil	4
• Glyceril stearate	4
• Cetearyl alcohol	4
• Isopropil miristate	3.8
• Vitamin F	2.5
• Lactic acid	1.6
• Stearic acid	1
• Propil paraben	0.1
• Controx VP ®	0.03
Water phase (W)	
• Water	70.05
• Glycerine	4.87
• Potassium phytate	2.85
• Laurylate S-90	0.3
• Imidazolidinil urea	0.3
• Essence	0.3
• Methyl paraben	0.2
• Triethanolamine	0.1

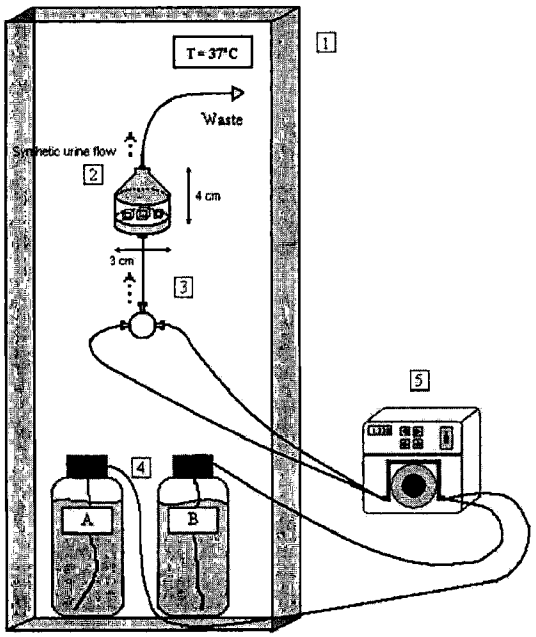

Figure 1. Diagram of the experimental flow system device used for crystallization studies with hydroxyapatite calculi. 1. Temperature-controlled chamber; 2. Flask containing the post-ESWL calculi fragments; 3. Three-way T mixing chamber of A and B solutions; 4. A and B solutions for artificial urine; 5. Peristaltic pump.

3.1.7. Calcium determination

Samples of kidneys were lyophilized and weighed. Dried tissues were digested in a mixture of 1:1 HNO_3:$HClO_4$ in a sand bath until the solution was clear. For calcium determination, digested samples were diluted with distilled water to a final volume of 10 mL. The concentration of calcium was determined by ICP-AES using the corresponding calibration curve.

3.1.8. Statistics

Results are expressed as means +/- standard error (SE.) One-way ANOVA was used to calculate significance of difference between groups. The Student *t*-test was used to assess differences of means. Conventional Windows software was used for statistical computations. A value of $p < 0.05$ was considered as being statistically significant.

3.2. *In vitro* studies

A collection of 24 spontaneously passed post-ESWL fragments of HAP calculi was collected the same day after ESWL application. The fragments were selected by combination of optical stereomicroscopy, infrared spectrometry and scanning electron microscopy (SEM) equipped with an energy dispersive X-ray analyzer (EDS) (25). All the selected fragments had similar morphology being this representative of that observed in the majority of spontaneously passed post-ESWL HAP pure renal calculi fragments.

The size of the selected fragments oscillated between 2-4 mm. Each of the four temperature-controlled (37°C) hermetic flow chambers (3 cm diameter and 4 cm high) contained three fragments of a HAP calculus with a total of 12 fragments used in each set of the experiments comprised of pH = 6.5 and normocalciuria ($[Ca^{2+}]$ = 3.75 mM) and pH = 6.5 and hypercalciuria ($[Ca^{2+}]$ = 6.25 mM). The fragments were placed in the experimental chamber without any previous pre-treatment process. Synthetic urine (26) was introduced into the flow chambers, freshly prepared, by a multichannel peristaltic pump, with a rate of 750 mL/day through the bottom of the flasks (Figure 1). The system was operating for different duration that allowed the growth of new crystals on the fragments. Growth of the fragments was assessed by weight increase and normalized by using the relative mass increase. In one set of experiments, the system ran for 48 hours under conditions of hypercalciuria/normooxaluria ($[Ca^{2+}]$ = 6.25 mM, [Oxalate] = 0.28 mM). In a second set of experiments, the system was allowed to run for 192 hours under conditions of normocalciuria/ normooxaluria ($[Ca^{2+}]$ = 3.75 mM, [Oxalate] = 0.28 mM).

3.2.1. Synthetic urine

Synthetic urine supersaturated with calcium oxalate was prepared by mixing equal volumes of solutions A and B (Table 2). The pH of both solutions was adjusted to 6.5. Solutions were stored for a maximum period of one week at 4 °C. Chemicals of reagent-grade purity were dissolved in deionized and redistilled water. All solutions were filtered through a 0.45 μm pore filter before use.

3.2.2. Effects of phytate

The inhibitory effects of phytate as sodium salt (Sigma-Aldrich, MO) on crystallization in synthetic urine were assessed in the concentration of 0.76-9.09 μM which is within the normal physiologic concentration of the urine (27).

4. RESULTS

4.1. *In vivo* studies

The calcium content of kidneys of the three studied groups (control, phytate non-treated and phytate treated) at the end of the experiment is shown in Figure 2

Table 2. Composition of synthetic urine (25)

Solution A (mM)		Solution B (mM)	
$Na_2SO_4 \cdot 10H_2O$	19.34	$NaH_2PO_4 \cdot 2H_2O$	15.45
$MgSO_4 \cdot 7H_2O$	5.93	$Na_2HPO_4 \cdot 12H_2O$	15.64
NH_4Cl	86.73	NaCl	223.08
KCl	162.60	$Na_2C_2O_4$	0.57

Different volumes of a 1 M calcium solution, prepared by dissolving calcium carbonate with hydrochloric acid were added to solution A to obtain a final calcium concentration of 3.75-6.25 mM.

Table 3. Phytate concentration in urine for the subject groups, after 31 days of AIN 76-A diet

	Groups		
	Control	Phytate non-treated	Phytate treated
Phytate concentration in urine (mg/L)	0.08 +/- 0.03 (n = 6)	0.09 +/- 0.03 (n = 6)	36.15 +/- 7.26[1,2] (n = 6)

[1]: $p < 0.001$ phytate treated versus control group, [2]: $p < 0.001$ phytate treated versus phytate non-treated group

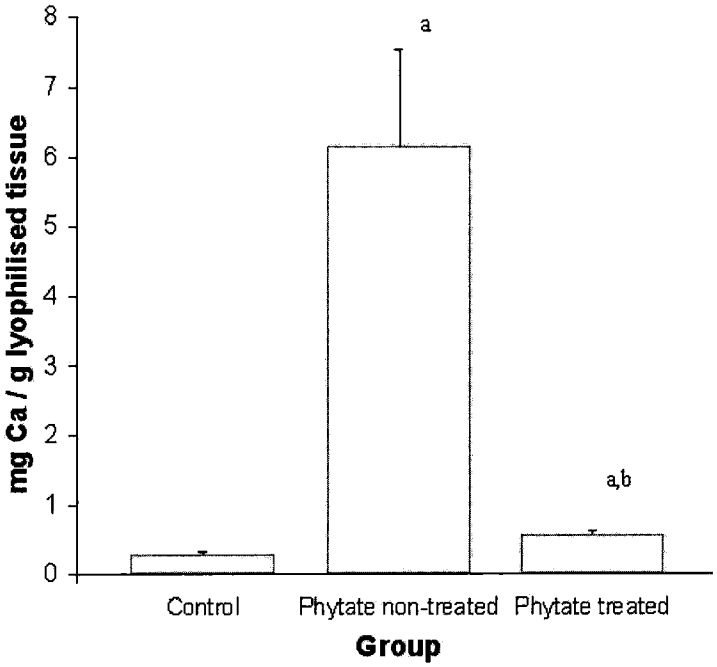

Figure 2. Calcium content in kidney of the subject groups (control, phytate non-treated, phytate treated). : $p < 0.005$ vs control group. : $p < 0.01$ vs phytate non-treated group.

and the concentration of phytate in urine after 31 days of AIN 76-A diet consumption is shown in Table 3. A significantly higher amount of calcium content was observed in the kidney of the phytate non-treated group (6.14 +/- 1.41 mg calcium / g dry kidney tissue) as compared with control (0.29 +/- 0.03 mg calcium/g dry kidney tissue) and phytate treated animals (0.56 +/- 0.06 mg calcium/g dry kidney tissue) (Figure 2). A significantly higher urinary excretion of phytate was detected in the phytate treated group as compared with control and phytate non-treated groups (Table 3).

As is shown in Figure 3, only phytate non-treated rats displayed significant level of calcium deposits in kidneys. The calcium deposits were assessed from histological analysis to be absent in control and phytate treated rats (Figure 3A) and to be present at a high level in phytate non treated rats. In this group, intratubular calcium deposits (Figure 3B) and calcium deposits in blood vessels (Figure 3C) and calcified areas on papillae (Figure 3D) were also observed.

4.2. *In vitro* studies

In the *in vitro* conditions (normophosphaturic, normooxaluric and pH = 6.5 synthetic urine), fragments of HAP calculi exhibited an important capacity to induce the growth of calcium salts on their surface. Thus, in normocalciuric conditions ($[Ca^{2+}]$ = 3.75 mM), COM crystals developed, at a rate of 0.36 +/- 0.10 µg/h per mg of HAP calculus fragment (Figure 4A, t = 192 h). Using hypercalciuric urine ($[Ca^{2+}]$ = 6.25 mM) brushite crystals were mainly developed, but also calcium oxalate dihydrate (COD) and HAP crystals were observed (Figure 4B, 4C, t = 192h). The overall growth rate in these conditions was 1.87 +/- 0.22 µg/h per mg of HAP calculus fragment. Presence of 1.5 mg/L phytate inhibited the development of COM

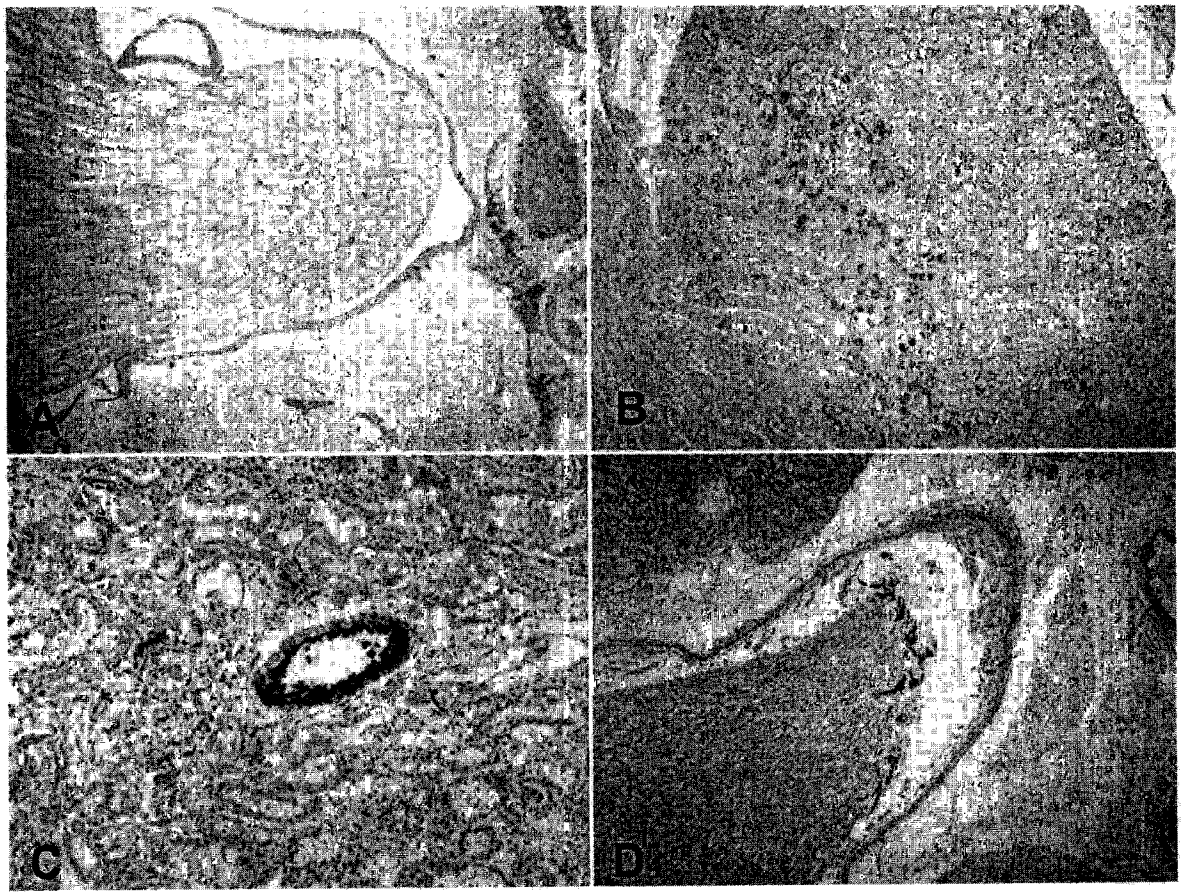

Figure 3. Sections of kidneys of the subject groups: A. Lack of calcium deposits in phytate treated rats (original magnification x 4) and control rats (not shown). B. Intratubular calcium deposits in phytate non-treated kidneys (original magnification x 4). C. Calcium deposits in blood vessels in phytate non-treated kidneys (original magnification x 20) D. Calcified areas in papillary region in phytate non-treated kidneys (original magnification x 4).

crystals on HAP calculi fragments in normocalciuric conditions (Figure 5A). In hypercalciuric conditions, presence of 6.0 mg/L phytate was necessary to inhibit crystal development (Figure 5B).

5. DISCUSSION

The findings reported here show that animals treated with D vitamin and nicotine develop calcified kidney deposit in renal tubules, blood vessels and on papillary epithelium. A significant number of papillary renal calculi (39.2%) initially develop on a subepithelial calcification deposit (Randall's plaque) that erodes the epithelium and gains direct contact with the urine. However, not all papillary calculi develop on a hydroxyapatite plaque and in 60.7% of these calculi no hydroxyapatite can be detected in the origin zone (1). In humans, there are different regions of kidney tissue calcification such as coarse focal deposits in the papillary region, and calcifications around the loops of Henle which are seen at all ages. The calcifications present at the boundary of the inner and outer medullary region are associated with degenerative changes, aging and arteriolar disease (7, 10 ,28). Development of these calcified areas appears to depend on injury or a pre-existing lesion such as papillary necrosis which leads to intratubular calcium phosphate deposits (29). Papillary calcification can be induced in rats with a combination of aspirin and sodium saccharin (30,31) and calcification of the vasa recta can be induced by long term phenacetin (32). Other studies demonstrated that high doses of phenylbutazone, oxyphenbutazone and indomethacin in rats leads to tubular necrosis in the lower nephron and causes calcification (33). Renal papillary necrosis and calcification also develop in diabetes mellitus (34). Hyperoxaluria which leads to hydroxyapatite tubular deposits in the lumen of collecting ducts and calcium oxalate monohydrate crystal deposits on the papillary tips is a precursor to papillary stones (9,35-40). The in vitro studies presented here, demonstrate that HAP fragments in contact with normocalciuric/normooxaluric urine induce COM crystal on their surfaces. Although this mechanism might be important to formation of some renal calculi, they are not the cause of all calculi since HAP is only found in 39.2 % of all COM papillary calculi (1). In the other cases, the nidus might be formed on sites with altered (damaged or just slightly injured) epithelium (2,3).

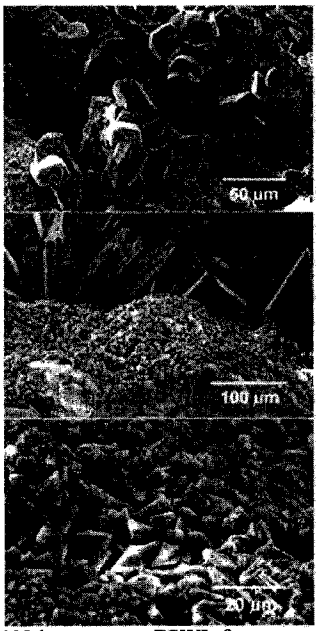

Figure 4. A. Formation of COM crystals after 192 hr on a post-ESWL fragment of a HAP renal calculus in normocalciuric (3.75 mM) and normooxaluric (0.28 mM of oxalate) synthetic urine (pH = 6.5) *in vitro*. B. Brushite, HAP and C. Formation of COD crystals after 192 h on post-ESWL fragments of HAP renal calculi in hypercalciuric (6.25 mM) and normooxaluric (0.28 mM of oxalate) synthetic urine (pH = 6.5) *in vitro*.

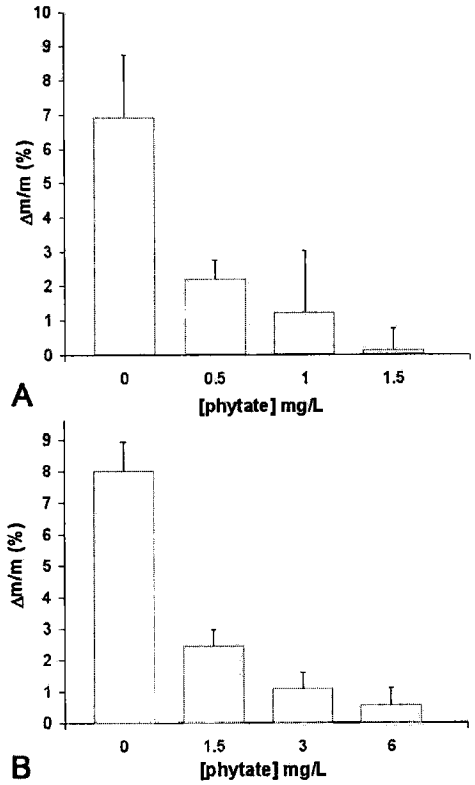

Figure 5. Effect of phytate on the increase of relative weight of post-ESWL fragments of HAP renal calculi maintained in normooxaluric ([oxalate] = 0.28 mM) synthetic urine at pH = 6.5. Values are means of 12 fragments ± SE. Normocalciuric urine ($[Ca^{2+}]$ = 3.75 mM). System kept working for 192 hours, Hypercalciuric urine ($[Ca^{2+}]$ = 6.25 mM). System kept working for 48 hours.

Although, the development of tissue calcification depends on a preexisting injury which acts as an inducer, continuation of this process is subject to modulators and/or the deficit of crystallization inhibitors. For example, some carboxyproteins act as osteopontin, bind HAP, signal and recruit macrophages that remove these calcifications or prevent their progression (41-46). The crystallization inhibitory action avoiding HAP development (nucleation and growth) has been attributed to low molecular weight compounds such as pyrophosphate, magnesium and phytate (47-49). Here, we show that phytate can act as crystallization inhibitor in both, the intrapapillary tissue and in urine. The *in vitro* results show that phytate prevents the development of calcifications on HAP at concentrations similar to those found in real urine (27).

The phytate levels found in tissues and blood in mammals clearly depend on dietary intake and these levels correlate with that found in the urine (12, 27, 33). Here, we show that phytate can be also readily absorbed from skin and might be clinically useful route of administration in subjects who are prone to the development of renal calculi.

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Phytate (*Myo*-inositol hexakisphosphate) inhibits cardiovascular calcifications in rats

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1. ABSTRACT

Calcification is an undesirable disorder, which frequently occurs in the heart vessels. In general, the formation of calcific vascular lesions involves complex physicochemical and molecular events. Calcification (hydroxyapatite) is initiated by injury and is progressed by promoter factors and/or the deficit of inhibitory signals. *Myo*-inositol hexakisphosphate (phytate, $InsP_6$) is found in organs, tissues and fluids of all mammals and exhibits an important capacity as a crystallization inhibitor of calcium salts in urine and soft tissues. The levels found clearly depend on the dietary intake but it can also be absorbed topically. In this paper, the capacity of $InsP_6$ as a potential inhibitor of cardiovascular calcifications was assessed in Wistar rats. Three groups were included, a control group, an $InsP_6$ treated group (subjected to calcinosis induction by Vitamin D and nicotine and treated with standard cream with a 2% of $InsP_6$ as potassium salt) and an $InsP_6$ non-treated group (only subjected to calcinosis induction). All rats were fed AIN 76-A diet (a purified diet in which $InsP_6$ is undetectable). Animals were monitorized every 12 hours. After 60 hours of calcinosis treatment, all rats of the $InsP_6$ non-treated group died and the rest were sacrificed. Aortas and hearts were removed. A highly significant increase in the calcium content of aorta and heart tissue was observed in the $InsP_6$ non-treated rats (21 +/- 1 mg calcium / g dry aorta tissue, 10 +/- 1 mg calcium / g dry heart tissue) when compared with controls (1.3 +/- 0.1 mg calcium / g dry aorta tissue, 0.023 +/- 0.004 mg calcium / g heart dry tissue) and $InsP_6$ treated (0.9 +/- 0.2 mg calcium / g dry aorta tissue, 0.30 +/- 0.03 mg calcium / g dry heart tissue) animals. Only $InsP_6$ non-treated rats displayed important mineral deposits in aorta and heart. These findings are consistent with the action of $InsP_6$, as an inhibitor of calcification of cardiovascular system.

2. INTRODUCTION

Calcification is an undesirable disorder frequently observed in the cardiovascular system, where it alters blood-vessel flexibility and promotes thrombosis and arterial rupture (1-3). When it appears in cardiac valves it is associated to several disorders that, if uncorrected, can lead to heart failure and death (4).

At present many identified risk factors for coronary artery calcifications as end-stage renal disease (5), advanced age (6), elevated plasma cholesterol (7), diminished high-density lipoprotein cholesterol (7), cigarette smoking (8), elevated blood pressure (9), obesity (7), diabetes (8) and elevated triglicerides (7) are known. Nevertheless, although vascular calcification may appear to be a uniform response to different types of vascular injury, it is a complicated disorder, with overlapping yet distinct unknown mechanisms of initiation and progression. In general, the formation of calcific vascular lessions appears to involve different complex physicochemical and molecular biological principles. Thus, it seems that the preexistence of an injury acting as inductor (heterogeneous nucleant) of the calcification (hydroxyapatite) is necessary and its progression would depend on the presence of other promoter factors (as hypercalcemia, hyperphosphatemia, etc.) and/or the deficit of inhibitory factors.
Some inhibitors of vascular tissue mineralization have been described, thus a candidate molecule seems to be a matrix gamma-carboxyglutamic acid (Gla) protein, a mineral binding protein (9), although the action of this protein appears to be also related to other complex cellular process linked to ossification processes (10). Phytate (*myo*-inositol hexakisphosphate, $InsP_6$) is a molecule found in abundance in plant seeds and also found in all mammalian organs, tissues and fluids but at significantly low concentrations (11,12). The levels found in blood and mammalian tissues clearly depend on its dietary intake (11,12), furthermore it has been demonstrated that this molecule is also topically absorbed (13) and it exhibits an important capacity as a crystallization inhibitor of calcium salts in urine and soft tissues (14,15). For this reason the aim of the present paper is to evaluate the capacity of phytate as a feasible inhibitor of cardiovascular calcifications.

3. MATERIALS AND METHODS

3.1. Animals, diets and treatments

Eighteen male Wistar rats of approximately 250 g from Harlan Iberica s.l. (Barcelona, Spain) were acclimated in the course of 7 days to our animal house. Animals were kept in Plexiglas cages (three animals per cage) at a temperature of 21 +/- 1 °C and relative humidity of 60 +/- 5 % with a 12-h on-off light cycle. After this period, animals were randomly assigned into three groups of six rats respectively. Control group (it was subjected to placebo calcinosis induction), $InsP_6$ treated group (it was subjected to calcinosis induction and pre-treated with $InsP_6$ cream) and $InsP_6$ non-treated group (it was only subjected to calcinosis induction). All rats were fed with AIN 76-A diet (Ssniff Especialdiatën GmbH, Soest, Germany), a purified diet in which $InsP_6$ is undetectable. The procedures used in this experiment were carried out according to the Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes and official permission to perform this animal experiment was obtained from the Bioethical Committee of our University.

3.2. $InsP_6$ cream pre-treatment

After a period of 16 days consuming AIN 76-A diet, the urinary $InsP_6$ became undetectable. Then $InsP_6$ treated group was topically subjected once a day until the end of the experiment (20 days) with 4 g of a standard cream with a supplement 2.0 % of $InsP_6$ as potassium salt used in previous work (13). The surface of treatment was about 50 cm^2. The application area was located on the back skin of the animal which was previously shaved using an *electric shaver* (each 4 days).

3.3. Calcinosis induction

After a period of 16 days with cream pre-treatment, the $InsP_6$ treated group and non-treated group were subjected to calcinosis induction and control group only to placebo calcinosis induction. Calcifications were induced according to P. Kieffer et al. (16) by injecting 300.000 IU/kg i.m. of vitamin D_3 (supplied by Fort Dodge Veterinaria, S.A., Girona, Spain) and 25 mg/kg p.o. of nicotine (as a solution 5 g/L of (-)-nicotine hydrogen tartrate salt supplied by Sigma Aldrich, Steinheim, Germany). The nicotine administration was repeated ten hours later. Control group received instead vitamin D_3 and nicotine, 0.15 M NaCl i.m. and destilled water respectively.

3.4. Monitorization and sample intake

The animals were monitorized every 12 hours. After 60 hours of calcinosis treatment, all rats of the $InsP_6$ non-treated group died and the rest of rats were sacrificed. Aortas and hearts were removed.

3.5. Histological analysis

Histological analysis of the aorta and heart calcifications was carried out on 10% buffered formalin fixed tissues. Tissues were first placed in 10% buffered formalin and fixed for 24 h at room temperature. Tissues were embedded, sectioned (sections of 4μm) and stained by hematoxiline-eosine. For histological analysis the section of all tissues was examined by an experimented pathologist. The calcium deposits presence was estimated semiquantitatively (in the transverse section) by absent, low, moderate or high notation.

3.6. $InsP_6$ determination

Samples of 24-h urine were collected at day 15 of cream pre-treatment to evaluate $InsP_6$ excretion by using a metabolic cage (Tecniplast, Gazzada, s.a.r.l., Italy).

The determination of $InsP_6$ levels in urine samples was performed using an analytical methodology based on column separation and following total phosphorus determination by inductively coupled plasma atomic emission spectrometry (ICP-AES) (17). This methodology allows a measurement of total $InsP_6$ with a detection limit of 60 μg/l.

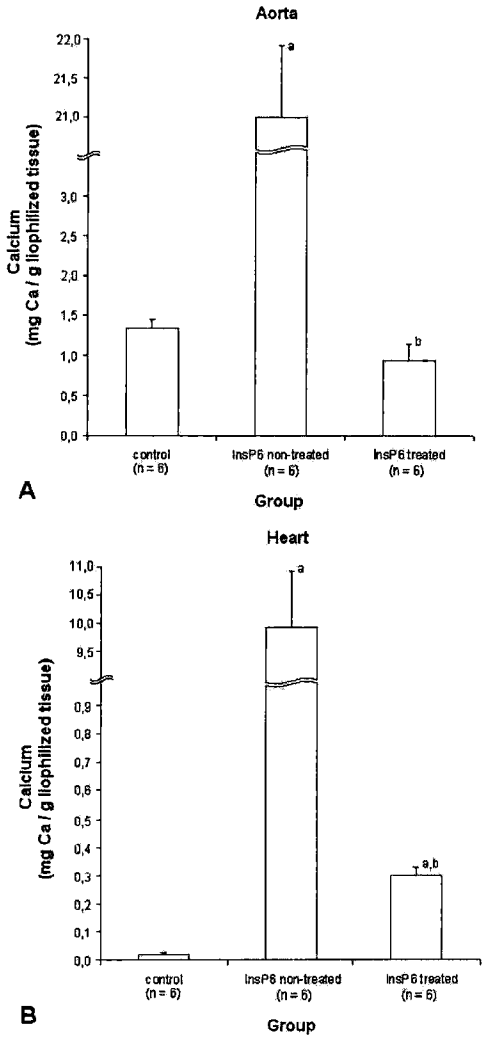

Figure 1. Calcium content in different tissues by the three studied groups (control, $InsP_6$ non-treated, $InsP_6$ treated): **a.** Aorta; **b.** Heart. a: $p < 0.001$ vs corresponding control group, b: $p < 0.001$ vs corresponding $InsP_6$ non-treated group

5.0 ml of urine (acidified with HCl 1:1 until pH = 3-4) was transferred to a column containing 0.2 g of anion exchange resin (the inner diameter was 4 mm). The first eluate was discarded, then the column was washed with 50 ml of HCl 50 mM. The second eluate was discarded. Then, the column was washed with 3.0 ml of HNO_3 2 M. The determination of $InsP_6$ was carried out through direct phosphorus analysis of this last eluate by ICP-AES using the corresponding calibration curve.

The ICP-AES conditions used were the following: outer argon flow 15 l/min, auxiliar argon flow 1 l/min, inner argon flow 1 l/min, nebulizer uptake rate 1 ml/min and wavelenght 213.618 nm.

3.7. Calcium determination

The samples of aortas and hearts were lyophilised and weighted. The residues were digested with 1:1 HNO_3:$HClO_4$ mixture in a sand bath until the solution was clear. For calcium determination, digested samples were diluted with distilled water until a final volume of 10 ml. The concentration of calcium was determined by inductively coupled plasma atomic emission spectrometry (Perkin-Elmer S.L., Spain) using the corresponding calibration curve.

3.8. Statistics

Values in the table and figures are expressed as mean +/- (SE). One-way ANOVA was used to calculate significance of difference between groups. The Student *t*-test was used to assess differences of means. Conventional Windows software was used for statistical computations. A value of $p < 0.05$ was considered to assess statistical significance.

4. RESULTS

The calcium accumulation in aorta and heart tissues by the three studied groups (control, $InsP_6$ treated and $InsP_6$ non-treated) is shown in Figure 1 and phytate concentration in urine after 15 days of cream pre-treatment is shown in Table 1. As can be observed in Figure 1, a highly significant increase in the calcium content of the two studied tissues was observed in the $InsP_6$ non-treated rats (21 +/- 1 mg calcium / g dry aorta tissue, 10 +/- 1 mg calcium / g dry heart tissue) when compared with controls (1.3 +/- 0.1 mg calcium / g dry aorta tissue, 0.023 +/- 0.004 mg calcium / g dry heart tissue) and $InsP_6$ treated (0.9 +/- 0.2 mg calcium / g dry aorta tissue, 0.30 +/- 0.03 mg calcium / g dry heart tissue) animals. No significant differences were observed in aorta calcium content between the $InsP_6$ treated rats and controls. Significant differences between heart calcium content of $InsP_6$ treated rats and control rats were also observed. In Table 1 it can be appreciated that highly significant urinary excretion of $InsP_6$ was observed in the $InsP_6$ treated rats when compared with controls and $InsP_6$ non-treated rats.

As it is shown in Figures 2 and 3, only non-treated $InsP_6$ rats displayed important mineral deposits in aorta and heart. The histological analysis of calcium deposits of aortas and hearts were estimated as absent in control and $InsP_6$ treated tissues and as severe in $InsP_6$ non-treated tissues. The calcium deposits in aorta were observed predominantly in the intima and the intern vessel layers. The heart calcium deposits were observed in the walls of coronary vessels and in the adjacent myocardium. Also they presented myocardium necrosis and inflammation (heart attack).

5. DISCUSSION

As it is commented in the introduction section, the development of undesirable hydroxyapatite deposits (calcifications) in the cardiovascular system is a consequence of unbalance between promoter factors (injuries, hypercalcemia, hyperphosphatemia) and inhibitory factors (default of cellular defences, deficit of crystallization inhibitors).

Table 1. Phytate ($InsP_6$) concentration in urine for the different groups after 15 days of cream pre-treatment

	Control	**$InsP_6$ non-treated**	**$InsP_6$ treated**
$InsP_6$ concentration in urine (mg/L)	0.08 +/- 0.03 (n = 6)	0.09 +/- 0.03 (n = 6)	36.15 +/- 7.26 [1,2] (n = 6)

1: $p < 0.001$ vs control group, 2: $p < 0.001$ vs $InsP_6$ non-treated group

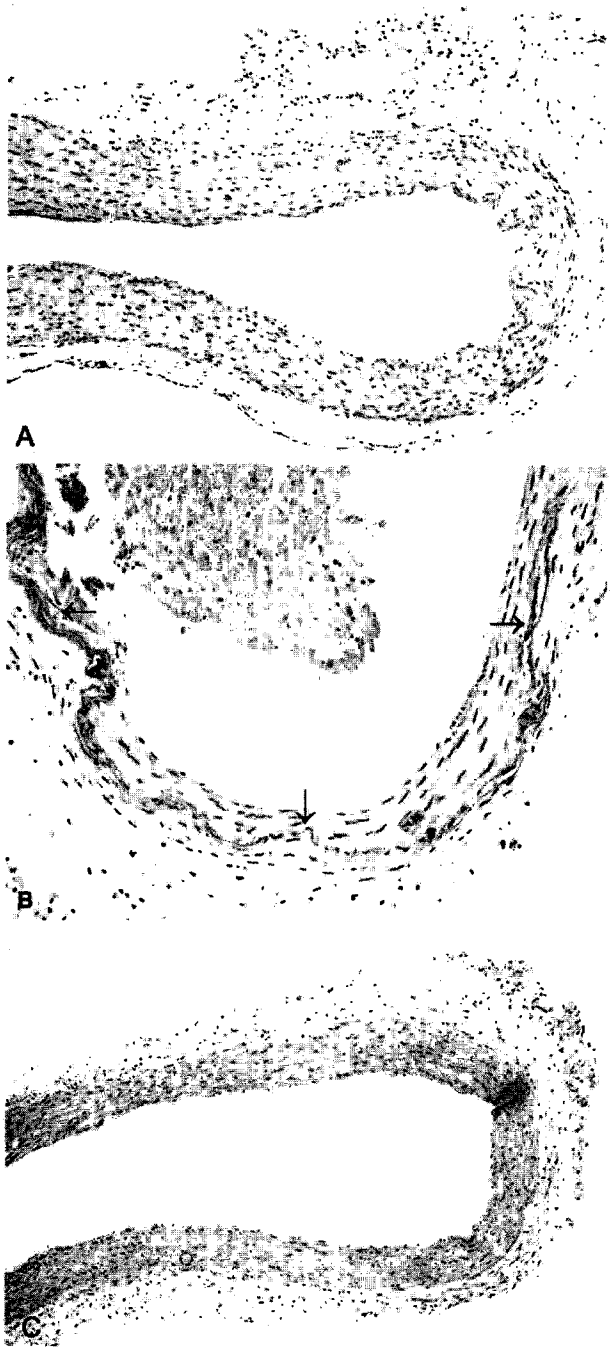

Figure 2. Images of sections of aortas by the three studied groups: **a.** control (original magnification x 10); **b.** $InsP_6$ non-treated (original magnification x 20); **c.** $InsP_6$ treated (original magnification x 10). The arrows show extensive calcifications.

Figure 3. Images of sections of hearts by the three studied groups: **a.** control (original magnification x 4); **b.** $InsP_6$ non-treated (original magnification x 10); **c.** $InsP_6$ treated (original magnification x 4). The arrows show extensive calcifications.

A number of extracellular matrix proteins have been reported to inhibit mineralization, including osteocalcin, albumin, osteopontin and matrix gamma-carboxyglutamic acid (Gla) protein (9,16,18-20). *In vitro* experiments demonstrate some activity of these proteins as crystallization inhibitors. Thus, osteopontin has been reported to inhibit *de novo* mineral formation by blocking crystal growth rather than hydroxyapatite nucleation (19,21,22) and can also promote cell adhesion and migration (23). Otherwise, also promoter activity of hydroxyapatite development has been assigned to these proteins or their analogs due to their nucleating activity (24-26). Nevertheless, a clear action of some of these proteins regulating the cellular activity on calcification processes has been described. Hence, osteocalcin is a Gla-containing protein with a potential function as an inhibitor of osteoblast activity (27). In the rapidly calcifying bone of newborn rats matrix Gla protein levels are about 130% higher than those in adult rats (28). Hence it seems that extracellular protein matrix has an important role as signaling agents in the control of cellular processes associated to the tissue calcification, rather than a typical action as crystallization inhibitors.

$InsP_6$ (phytate) has demonstrated a powerful capacity as crystallization inhibitor of hydroxyapatite in *in vitro* experiments (29) and in no cases it has demonstrated a promoting capacity. The levels found in mammals clearly depend on its dietary intake (11,12) being this molecule also topically absorbed (13) as it is also demonstrated by the presented results, correlating the amounts found in blood and tissues with the urinary values (11,12). On the other hand, this is a molecule of low molecular weight to which no signaling capacity of cells implied in calcification processes has been assigned. Thus, considering the results obtained in this paper, the clear action of $InsP_6$ avoiding the calcification of cardiovascular system must be attributed to its capacity as crystallization inhibitor. Consequently, this could be another important factor linked to cardiovascular calcifications that need further studies to be able to evaluate its authentic dimension. In this aspect, it is interesting to comment that culture of normal aortas with alkaline phosphatase resulted in hydroxyapatite calcification of the elastic lamina. This was not due to dephosphorylation of osteopontin and calcification was not increased in aortas from osteopontin-deficient mice. The inhibitor was identified as pyrophosphate (30), which is another well known crystallization inhibitor of hydroxyapatite in *in vitro* experiments (29,31). Consequently, when hydroxyapatite inhibitory factors are considered, mechanisms of cellular defence to such processes and authentic crystallization inhibitory activity of some molecules in front of hydroxyapatite development should probably be distinguished.

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Key Words: Phytate, Crystallization Inhibitor, Cardiovascular Calcifications, Heart, Vessel

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Study of the Absorption of *Myo*-Inositol Hexakisphosphate ($InsP_6$) through the Skin

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Recently, some properties of *myo*-inositol hexakisphosphate ($InsP_6$) are related to its dermatological use as discolouring agent, on preventing calcinosis cutis or due to its important role on premature aging. Some studies also seem to demonstrate a capacity of $InsP_6$ to inhibit skin cancer. In this paper, a first study of the absorption of $InsP_6$ through the skin is developed. Due to the correlation between $InsP_6$ absorption and its urinary excretion, these last values were used to evaluate this process. It was found that using a moisturizing cream as vehicle, the $InsP_6$ sodium salt was absorbed at significantly higher amounts than the $InsP_6$ calcium–magnesium salt. Maximum $InsP_6$ urinary concentrations were observed approximately at 14 d of 2% $InsP_6$ topical cream application, and gave 66.35±5.49 mg/l urinary $InsP_6$ when the sodium salt was used and 16.02±2.61 mg/l urinary $InsP_6$ when the calcium–magnesium salt was applied. When the $InsP_6$ topical cream administration ceased, the $InsP_6$ urinary excretion fell dramatically approximately during a period of 10 d. From these results, it can be deduced that by topical administration $InsP_6$ can achieve important concentrations in tissues and biological fluids, this demonstrating that it is possible to propose the topic use as a new $InsP_6$ administration route.

Key words $InsP_6$ skin absorption; urinary excretion; cream application; dermatological use

Myo-inositol hexakisphosphate ($InsP_6$) is an abundant component of plant seeds. In whole grain cereals it ranges from 1.5 to 6.4% and it is mostly associated with calcium and magnesium ions, the so-called phytin.[1,2] Recently it was found that $InsP_6$ is also present in all mammalian organs, tissues and fluids but at significantly low amounts. Moreover, it was demonstrated that the levels found in biological fluids (blood, urine, interstitial liquid) and mammalian tissues clearly depended on the dietary intake.[3—5]

Diverse studies performed by Mellanby demonstrated that a high $InsP_6$ content in some diets, as sodium salt, reduced calcium absorption and induced rickets.[6] Ever since up to now, several studies have attributed "anti-nutritional" properties to phytate.[7—11] Nevertheless, other studies[12—16] have shown that those findings are not quite so clear and simple as mentioned. Moreover, from the 1980s to the present, important physiological functions of $InsP_6$ have been suggested as its properties as an antioxidant[17,18] and its role in colon cancer prevention.[19,20] The $InsP_6$ present in urine and biological fluids also exhibited an important role in preventing pathological calcifications as renal calculi[21—23] or calcinosis cutis,[24] due to its powerful capacity to act as crystallization inhibitor of calcium salts.

Finally, the most recent observations about the properties of $InsP_6$ are related to its dermatological use. The majority of those applications are referred to their important action on premature aging or as discolouring agent of the skin.[25] Also some studies seem to demonstrate a capacity of $InsP_6$ to inhibit skin cancer.[26,27] In the present paper a first study of the absorption of $InsP_6$ through the skin is developed.

MATERIALS AND METHODS

Animals, Diets and Experimental Design Twenty-four female Wistar rats of approximately 225 g from Harlan Iberica s.l. (Barcelona, Spain) were acclimated in the course of 7 d to our animal house. Animals were kept in Plexiglas cages (two animals per cage) at a temperature of 21±1 °C and relative humidity of 60±5% with a 12-h on–off light cycle. After this period, animals were randomly assigned into four groups of six rats respectivelly. Rats were fed with 4068.02 Reference Diet (HopeFarms BV, Woerden, The Netherlands), a synthetic purified diet (Table 1) in which $InsP_6$ is undetectable.

After a period of 16 d consuming such diet, during which the urinary $InsP_6$ became undetectable, rats were topically treated once a day with 4 g of a standard cream with a supplement of 0.4, 1.2 and 2.0% of $InsP_6$ as sodium salt or 2.0% of $InsP_6$ as calcium magnesium salt (phytin). The surface of treatment was about 50 cm^2. The application area was located on the back skin of the animal and was previously shaved using an electric shaver (each 4 d). During cream treatment animals were located individually to avoid licking cream. pH of all creams was adjusted to 4—4.5 (see Table 2). Samples of 24-h urine were collected at days 0, 7 and 14 to evaluate $InsP_6$ excretion. After 14 d of treatment, rats treated with the standard cream containing 0.4 and 1.2% of $InsP_6$ as sodium

Table 1. Composition of 4068.02 Reference Diet, a Synthetic Purified Diet in Which $InsP_6$ Is Undetectable

Ingredient	% in diet
Acid casein	20
Corn starch	10
Cellulose	5
Soybean oil	5
Glucose	52.8
$CaPO_4 \cdot 2H_2O$	1.3
$CaCO_3$	1
KH_2PO_4	0.40
KCl	0.70
NaCl	0.30
$MgSO_4 \cdot 7H_2O$	0.40
MgO	0.20
DL-Methionine	0.20
Choline choride	0.40
Standard vitamin mix	1
Standard mineral mix	1

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Table 2. Composition of Moisturizing Creams Used

Component	Percentage composition			
	0.4% $InsP_6$ (sodium salt) enriched cream	1.2% $InsP_6$ (sodium salt) enriched cream	2.0% $InsP_6$ (sodium salt) enriched cream	2% $InsP_6$ (as phytin) enriched cream
$InsP_6$ salt	0.6	1.7	2.9	2.6
Almond oil	4	4	4	4
Thomil ISF	3.8	3.8	3.8	3.8
Stearic acid	1	1	1	1
Lactic acid	1.6	1.6	1.6	1.6
Vitamin F 09929	2.5	2.5	2.5	2.5
Monestriol GAE	4	4	4	4
Propyl Paraben	0.1	0.1	0.1	0.1
Pumol 1618	4	4	4	4
Controx VP	0.03	0.03	0.03	0.03
Water	72.3	71.2	70	70.3
Triethanolamine	0.1	0.1	0.1	0.1
Laurylate S-90	0.3	0.3	0.3	0.3
Glycerine 3699 USP	4.87	4.87	4.87	4.87
Methyl Paraben	0.2	0.2	0.2	0.2
Abiol	0.3	0.3	0.3	0.3
Essence	0.3	0.3	0.3	0.3

salt were sacrified, whereas the treatment was mantained for rats treated with the standard cream containing 2.0% of $InsP_6$ as sodium salt or calcium/magnesium salt, until $InsP_6$ urinary excretions became constant (34 d). Then, the cream application ceased but collection of 24-h urine samples continued until $InsP_6$ urinary levels decreased and became constant. When finishing the experiment, animals were sacrified. The procedures used in this experiment were carried out according to the Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes and official permission to perform this animal experiment was obtained from the ethical committee of our University.

$InsP_6$ Determination The determination of $InsP_6$ levels in urine samples was performed using an analytical methodology based on the determination of total phosphorus by atomic emission spectrometry ICP.[28] This methodology allows a measurement of total $InsP_6$ with a detection limit of 60 μg/l.

Procedure: 5 ml of urine (acidified with HCl 1 : 1 until pH=3—4) was transferred to a column containing 0.2 g of anion exchange resin (the inner diameter was 4 mm). The first eluate was discarded, then the column was washed with 50 ml of HCl 50 mM. The second eluate was discarded. Then, the column was washed with 3 ml of HNO_3 2 M. The determination of $InsP_6$ was carried out through direct phosphorus analysis of this last eluate by ICP-AES using the corresponding calibration curve.

The ICP-AES conditions used were the following: outer argon flow 15 l/min, auxiliar argon flow 1 l/min, inner argon flow 1 l/min, nebulizer uptake rate 1 ml/min and wavelenght 213.618 nm.

Statistics Values in the figures are expressed as mean (S.E.). The Student t-test was used to assess differences of means. Conventional Windows software was used for statistical computations. A value of $p<0.05$ was considered to assess statistical significance.

RESULTS

The obtained results about the topically treated rats with a cream containing different $InsP_6$ amounts and salts are shown in Figs. 1—3. As can be observed the $InsP_6$ sodium salt was absorbed at significantly higher amounts than the $InsP_6$ calcium–magnesium salt. Thus, after 7 d of topical cream application containing a 2% $InsP_6$, the sodium salt corresponded to 23.35±2.46 mg/l $InsP_6$ urinary excretion whereas the calcium–magnesium salt corresponded to a 11.75±3.96 mg/l $InsP_6$ urinary excretion. For this period of time, the $InsP_6$ excreted urinary amount of the sodium salt clearly depended on the $InsP_6$ cream concentration. Maximum $InsP_6$ urinary concentrations were observed approximately at 14 d of 2% $InsP_6$ topical cream application and gave 66.35±5.49 mg/l urinary $InsP_6$ when the sodium salt was used and 16.02±2.61 mg/l urinary $InsP_6$ when the calcium–magnesium salt was applied. When the $InsP_6$ topical cream administration ceased, the $InsP_6$ urinary excretion fell dramatically approximately during a period of 10 d.

DISCUSSION

In spite of some dermatological applications of $InsP_6$ being established at present,[25] and several topical $InsP_6$ based creams can be found in the market, no studies on the $InsP_6$ absorption through the skin can be found in the literature. In a recent paper, it was demonstrated that $InsP_6$ topically administered, notably and significantly reduced the development of subepithelial dystrophic calcifications in soft tissues,[29] this demonstrating that $InsP_6$ was unquestionably absorbed through the skin. The results of the present paper clearly demonstrated that $InsP_6$ was absorbed through the skin layers, crossing the epidermis and dermis, entering the blood stream and increasing the urinary excretion. It is interesting to compare the absorption through the skin with the absorption through the gastrointestinal tract. Thus, in both cases there seems to be an optimum dose, above which no

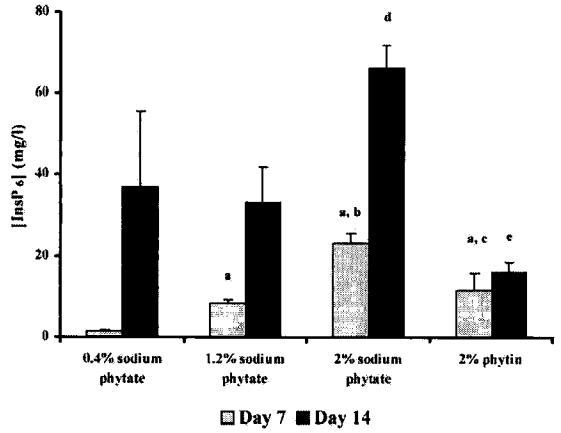

Fig. 1. Influence of $InsP_6$ Concentration in the Topically Applied Cream and $InsP_6$ the Salt Used on Urinary $InsP_6$ Concentration

[a] $p<0.05$ *vs.* 0.4% $InsP_6$ (day 7), [b] $p<0.05$ *vs.* 1.2% $InsP_6$ (day 7), [c] $p<0.05$ *vs.* 2.0% $InsP_6$ (day 7), [d] $p<0.05$ *vs.* 1.2% $InsP_6$ (day 14), [e] $p<0.05$ *vs.* 2.0% $InsP_6$ (day 14).

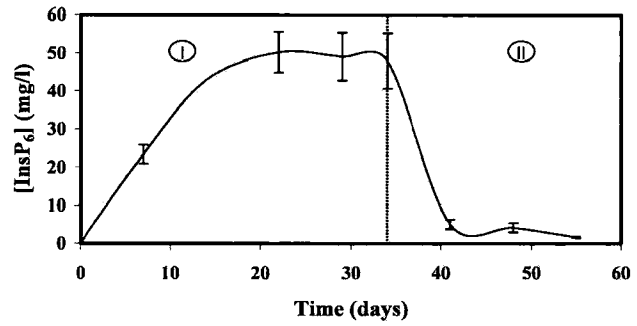

Fig. 2. $InsP_6$ Urinary Concentration in Rats

Section I: topically treated rats with a cream containing 2.0% of $InsP_6$ as sodium salt. Section II: the treated rats had stopped the topical cream application.

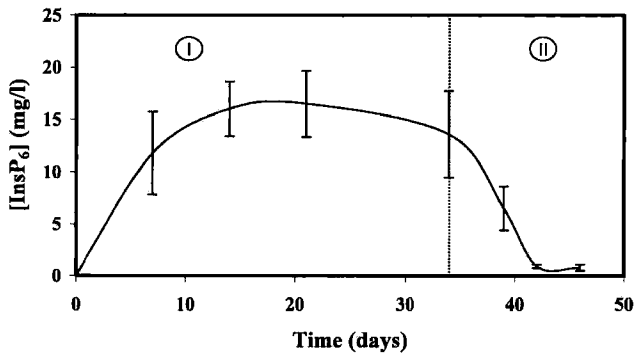

Fig. 3. $InsP_6$ Urinary Concentration in Rats

Section I: topically treated rats with a cream containing 2.0% of $InsP_6$ as phytin (calcium/magnesium salt). Section II: the treated rats had stopped the topical cream application.

further absorption occurs. Nevertheless, in the case of the gastrointestinal tract this amount was independent of the type of $InsP_6$ salt consumed[4] but in the case of the skin, the $InsP_6$ sodium salt was absorbed much more favorably than the $InsP_6$ calcium–magnesium salt, thus the $InsP_6$ urinary levels were triple when the sodium salt was used. On the other hand, it is important to emphasize that the attained $InsP_6$ urinary levels found when $InsP_6$ was administered orally were almost always inferior to those observed when $InsP_6$ was topically administered. Thus, maximum $InsP_6$ urinary levels found when $InsP_6$ was administered orally through the diet or using specific complements, in no case allowed to attain values superior to 6—7 mg/l $InsP_6$,[3] however through topical application urinary values of around 60 mg/l were achieved. This different behaviour of gastrointestinal and skin absorption could be in part explained considering that formation of insoluble no absorbable salts with divalent and trivalent cations and proteins in the gastrointestinal tract is more feasible due to the presence of food or food derivatives. In fact, from the results presented here it is observed that the $InsP_6$ sodium salt was clearly more favorably absorbed through the skin when compared with the calcium–magnesium salt.

From the presented results it can be deduced that by topical administration $InsP_6$ can achieve important concentrations in tissues and biological fluids, this demonstrating that it is possible to propose the topic use as a new $InsP_6$ administration route.

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Absorption of myo-inositol hexakisphosphate ($InsP_6$) through the skin in humans

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In this paper, we present a pilot study of the absorption of myo-inositol hexakisphosphate ($InsP_6$) through the skin in humans. We found that, after topical treatment with a 4% $InsP_6$ rich gel, $InsP_6$ urinary excretion increased 54% compared to the control situation (participants submitted to an $InsP_6$-poor diet for 15 days, $n = 6$), clearly demonstrating that $InsP_6$ is absorbed through the skin of humans. These results demonstrate the topical application as a suitable administration route of $InsP_6$ in humans.

Myo-inositol hexakisphosphate (phytate, $InsP_6$) is a molecule to which several beneficial properties have been recently attributed. Some of these properties are related to its dermatological use. Thus, it has been claimed the capacity of $InsP_6$ to inhibit skin cancer (Ishikawa et al. 1999; Gupta et al. 2003; Grases et al. 2005) and to avoid calcinosis cutis (Grases et al. 2005b). In spite of these facts, little is known about the dermal absorption of $InsP_6$ in humans. Previous studies demonstrated that absorption was observed when applying $InsP_6$ topically to rats, with urinary $InsP_6$ concentrations much higher than those found with $InsP_6$ ingestion (Grases et al. 2005b). It was also found that in rats, $InsP_6$ was absorbed through the skin using both a gel or a cream, demonstrating that its absorption is independent of the matrix used for the topical application (Grases et al. 2005b).

Twenty healthy volunteers (7 males and 13 females) were selected to study the dermal absorption of $InsP_6$ in humans. Due to the direct relation between serum $InsP_6$ concentration and its urinary excretion (Grases et al. 2001), this last parameter was used to evaluate $InsP_6$ dermal adsorption. The experiment had two phases. In the first one, all participants were submitted to an $InsP_6$-poor diet for 15 days (all types of integral cereals and integral cereal derivatives, integral rice, corn, legumes, all types of nuts and other vegetable seeds were totally excluded). It has been demonstrated that $InsP_6$ levels in biological fluids and mammalian tissues clearly depend on dietary intake, consequently these levels must be low after this first period. On the 15th day, early in the morning (7 : 00) volunteers voided the urine accumulated overnight in the bladder and after two hours fasting, urinary samples were collected (2 h-urine). Then, the subjects began the second phase of the experiment. They continued with an $InsP_6$-poor diet and were topically treated twice a day with 10 g of a standard moisturizing gel with a 4.0% content of $InsP_6$ as potassium salt (Table). The surface of treatment was about 1400 cm^2. Urinary samples were again collected at the 7th day of treatment to evaluate $InsP_6$ excretion (2 h-urine). The experimental procedure was approved by the bioethics committee of the University of Balearic Islands. The obtained results are shown in the Fig.. As it can be observed, after topical treatment with $InsP_6$ rich gel, $InsP_6$ urinary excretion increased by 54%. That result clearly demonstrated that $InsP_6$ was absorbed through the skin layers of humans, crossed the epidermis, arrived to the dermis, entered the blood stream and increased urinary excretion. From the presented results, it can be deduced that topical administration of $InsP_6$ to humans can increase its concentrations in tissues and biological fluids, this demonstrating that the topical application, can be proposed as a new administration route of $InsP_6$ in humans.

Table: Composition of the moisturizing gel containing 4% of phytate as potassium salt

Component	Percentage composition
Water	86.4
Propylene glycol	6
PNC 400	2
Astro C-40	0.2
Potassium phytate	5.4 (4% $InsP_6$)

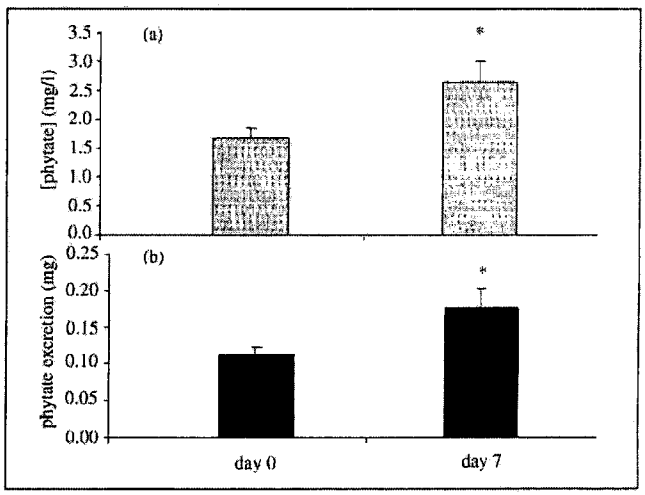

Fig.: (a) Urinary phytate concentration (mg/l) and (b) urinary phytate excretion (mg/2 h) at day 0 (before treatment) and at day 7 (after $InsP_6$ rich gel application). Values are mean of 20 subjects. Student's t-test was used to determine statistic significance between means. Error bars represent SE (* $p < 0.05$ vs day 0)

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SANIFIT
LABORATORIES

RELATIONSHIP BETWEEN PHYTATE AND VALVULAR HEART CALCIFICATIONS. FIELD STUDY TO DESIGN A CLINICAL ASSAY ABOUT AORTIC STENOSIS

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Valvular Heart calcification is a complex process initiated by an endothelial lesion that progresses due to several causes and it can origin valvular stenosis and, specifically, aortic stenosis with the implications that this fact implies. Myo-inositol hexaphosphate (phytate, InsP6) is present in organs, tissues and fluids of all mammalians and it shows an important capacity as a crystallization inhibitor of calcium salts in urine and soft tissues. Its levels only depend on the dietary intake and in previous studies with experimentation animals our group demonstrated its role as an inhibitor of vascular calcifications. In order to adequately design a clinical assay, we performed a field study to explore the relationship between phytate and vascular calcifications.

In this communication we report the preliminary results.

Material and methods: during the June of 2005, the echocardiograms performed due to any indications in the laboratory of echocardiography of Son Dureta Hospital were analysed, and the presence of valvular calcium was quantified (Rosenhek). The patients were included if they could and accepted to participate in the study and had at least 2 points in the scale of Rosenhek. Chronic or terminal renal insufficiency were excluded. Urinary phytate and serum / haematological parameters were determined. A survey of health and alimentary habits was performed.

Results: 138 patients with some degree of valvular calcification were included. Patients were classified as group 1 (slight valvular calcification) and group 2 (moderate or severe valvular calcification).

	Sex H NS	Age NS	BMI NS	AS P<0,0000	MRC P=0,018	Phytate P=0,065
Group 1	44 %	68,97±10,4	28,01±4,2	4,3 %	8,3 %	0,81 ± 1,09
Group 2	56 %	69,96±11,4	28,19±5,1	95,7 %	91,7 %	0,55 ± 0,49

M: man, BMI: body mass indez, AS: aortic stenosis, MRC: mitral ring calcification, phytate: niveles de fitato en orina ppm.

Urinary levels of phytate in both groups were notably lower to those considered as normal (2,94 ± 0.20 ppm in a study with healthy volunteers). Even though, there was a clear tendency of phytate levels to decrease with the increase of the degree of calcification. The same result was observed when classifying the patients according to the presence or absence of aortic stenosis.

(AS 0,54±0,45 vs no AS 0,77±0,9 p=0,09).

Other observations, as it had been previously described in the bibliography, the presence of antecedents of cancer was concentrated in the lower phytate values (23,4 % vs 5,6 % for a cut point lower than 0,3 ppm p=0,02).

Conclusions:

These results agree with the hypothesis of the role of phytate as an inhibitor of human valvular calcifications..